Synthesis, Characterization, and Biodegradation of Carboxymethylchitosan-*g*-Medium Chain Length Polyhydroxyalkanoates

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ABSTRACT: Grafting of medium chain length polyhydroxyalkanoates (mcl-PHA) produced by *Comamonas testosteroni* onto carboxymethylchitosan (CMCH) using ceric ammonium nitrate (CAN) as an initiator was carried out under nitrogen atmosphere in aqueous medium. The grafting composition was 2 g CMCH, 0.2*M* CAN, and 0.5 g mcl-PHA. The reaction was carried out at 40°C ± 1°C for 4.5 h, and reaction product was extracted by acetone precipitation. The CMCH-*g*-mcl-PHA copolymers were characterized by Fourier transform infrared spectroscopy, Thermogravimetric analysis, differential scanning calorimetry, and scanning electron microscopy. The data obtained showed successful grafting of mcl-PHA onto CMCH polymer. TGA results indicated that the graft was stable up to 380°C, and the solubility studies revealed a high % grafting efficiency. Biodegradation studies of the graft in terms of microbial growth, extracellular protein concentration, and % weight loss in the graft were carried out for 30 days using a bacterial isolate *Burkholderia cepacia* 202 and a fungal isolate *Aspergillus fumigatus* 202. 93% weight loss of the graft was obtained in case of *A. fumigatus* 202, whereas *B. cepacia* 202 showed 76% loss in weight of the graft.
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Key words: CMCH-*g*-mcl-PHA; FTIR; TGA; SEM; biodegradation

INTRODUCTION

Poly(3-hydroxyalkanoates) (PHAs) are a class of naturally occurring polyesters that are synthesized as carbon and energy reserve materials by a wide range of bacteria, usually under unbalanced growth conditions.[1] Because it is a truly biodegradable and excellent biocompatible material, it is suitable for two promising applications: one is as a viable candidate for relieving environmental concerns accused by disposal of nondegradable plastics; the other is to provide biomedical material such as surgical sutures, long-term carriers of drug, and tissue engineering. Two types of PHAs according to the length of the side chain are distinguished (i) short-chain length polyhydroxyalkanoates (scl-PHAs) and (ii) medium-chain length polyhydroxyalkanoates (mcl-PHAs). Scl-PHAs are too rigid and brittle and lack the superior mechanical properties required for biomedical and packaging film applications. In contrast, mcl-PHAs are elastomeric but have very low mechanical strength. Therefore, for packaging materials, biomedical applications, tissue engineering, and other specific applications, the physical and mechanical properties of these PHAs need to be diversified and improved. Many problems associated with the use of mcl-PHA might be alleviated by the use of its grafts or blends.[2]

Physical blending and chemical copolymerization of mcl-PHAs with suitable, well-defined polymers are the means to produce materials with desirable properties. Some of mcl-PHA blend systems have been studied to screen materials with improved properties.[2] Cakmakli et al.[3] have reported grafting reactions of poly(styrene peroxide) and poly(methyl methacrylate peroxide) onto unsaturated bacterial polyesters produced by *Pseudomonas oleovorans*. Studies on the synthesis of graft of polyhydroxyoctanoate (PHO) and chitosan by condensation reactions were carried out by Arslan et al.[4] Graft copolymerization is considered to be one of the most promising approaches to a wide variety of molecular designs leading to novel type of tailored hybrid polymeric materials.[5]

Chitosan [poly-β(1-4)-D-glucosamine] is a chiral material suitable for asymmetric separation of racemic mixtures and for biomedical applications. It also has applications in the food, medical, pharmaceutical, agricultural, and chemical industries.[6,7] Chitosan

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has functional groups like hydroxyl, amine, and amides, which can be chemically modified. It is brittle and is usually blended or grafted with other biocompatible polymers to modify its properties for biomedical applications like use as artificial actuator and tissue scaffold. PHAs, especially mcl-PHAs are elastomers, and so they may serve well as biocompatible natural plasticizers to reduce the stiffness of chitosan.

In this study, an approach to produce a novel copolymer of modified chitosan with mcl-PHA as side chains has been evaluated. Chitosan has been modified to carboxymethylchitosan (CMCH) and then grafted with mcl-PHA. So far, many grafting and blending reactions of PHA have been studied[2]; however, to our knowledge, this is the first report on grafting of mcl-PHA onto CMCH. In addition, the thermal and solubility properties of the graft were studied. These properties proved it to be an industrially viable polymer, which further motivated us to investigate its biodegradability.

EXPERIMENTAL

Materials

Chitosan (molecular weight 8.4×10^4; degree of deacetylation 85%) was provided by the Central Institute of Fisheries Technologies, India; Ceric ammonium nitrate (CAN) from S.D. Fine Chemicals, India. The medium used for inoculum development was Nutrient Broth (Hi-Media, India) and for PHA accumulation was Bushnell Haas Minerals (BHM) medium (Hi-Media, India). Coconut oil was purchased from the local market.

Mcl-PHA biosynthesis and extraction

Comamonas testosteroni was grown on 1% coconut oil in BHM medium. The production and extraction of mcl-PHA were carried out by using methods in the literature.[8]

Preparation of *O*-CMCH

O-Carboxymethylchitosan (CMCH) was prepared according to the method described by Chen and Park,[9] where chitosan (10 g), sodium hydroxide (13.5 g), and solvent isopropanol (100 mL) were suspended in a flask allowing chitosan to swell and alkalize at room temperature for 1 h. The monocholoroacetic acid (15 g) was dispersed in isopropanol and added to the reaction mixture drop-wise over 30 min and reacted for 4 h at 55°C. Then the reaction was stopped, and isopropanol was discarded. Ethyl alcohol (80%) was added, and the CMCH obtained as a solid product was separated by filtration. The graft was then rinsed with 80–90% ethyl alcohol to desalt and dewater followed by vacuum drying at 50°C.[10]

Grafting reaction

Two grams of CMCH, a predetermined amount of mcl-PHA (0.5 g) and 120 mL of double-distilled water were charged in a three-necked round-bottomed flask in a constant temperature water bath maintained at a 40°C. Nitrogen gas was bubbled for 30 min to remove the dissolved oxygen under stirring. CAN (0.2*M*) dissolved in 10 mL of 0.3*M* HNO_3 was slowly added to the three-necked flask to initiate graft copolymerization. The copolymerization reaction was carried out for 4.5 h. Reaction products were neutralized by 10% NaOH, precipitated in acetone, filtered, and washed with acetone and methanol : H_2O (90 : 10), so that all the unreacted CMCH and ceric salt were removed and dried under vacuum at 50°C. CMCH-*g*-mcl-PHA thus obtained was extracted with ethyl alcohol for 48 h and dried at 50°C for 24 h.

Polymer characterization

IR spectra of polymers were recorded with a Perkin-Elmer, Fourier transform infrared (FTIR) spectrometer using KBr pellets. The thermal stability of polymers was characterized by thermogravimetric analysis (TGA) (Universal V2.6D, TA Instruments). Differential scanning calorimetry of the graft was carried out using Thermal Analysis System (Perkin-Elmer TGA-7-DSC-PYRIS-1DTA-7).

Scanning electron microscopy of the polymers was carried out using SEM XL-Series from Philips (The Netherlands) at 15 kV. Solubility studies of the CMCH-*g*-mcl-PHA were examined by dissolving 10 mg of graft in various organic as well as inorganic solvents and keeping under shaking as well as static conditions for 24 h.

Biodegradation studies of the polymers

All three polymers namely mcl-PHA, CMCH, and CMCH-*g*-mcl-PHA were subjected to biodegradation studies using a bacterial culture *Burkholderia cepacia* 202 and a fungal culture *Aspergillus fumigatus* 202. For which, 1 g of the polymer was kept in a 500-mL Erlenmeyer flask, containing 200-mL BHMs medium. The flasks were inoculated with *B. cepacia* 202 (1.0 initial OD_{660}) and incubated at 37°C under static as well as shaking conditions (150 rpm). Similarly, flasks were inoculated with 10^6 spores/mL of *A. fumigatus* 202. Samples of 1.5 mL each were removed at an interval of 2 days for a period of 30 days. The samples were analyzed for growth (CFU/mL) and

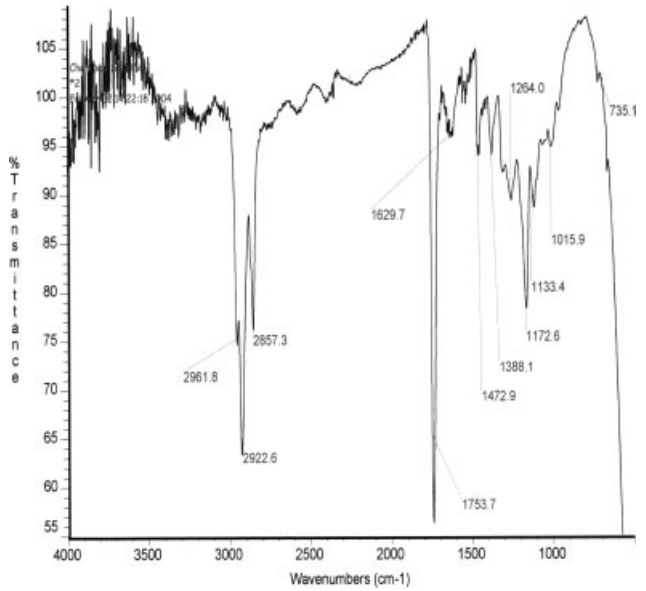

Figure 1 FTIR of mcl-PHA extracted from *Comamonas testosteroni*.

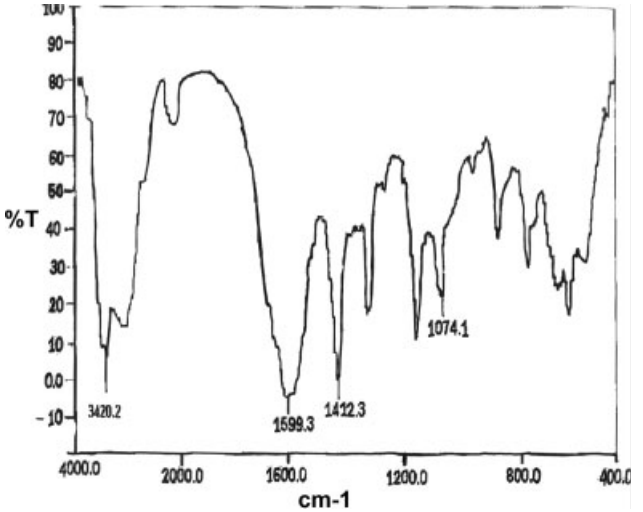

Figure 2 FTIR of CMCH.

extracellular protein concentration.[11] Percent weight loss of the polymer in each flask was determined after 30 days.[12,13]

RESULTS AND DISCUSSION

Although graft copolymerization of PHA is rather difficult due to its nonactive chemical structure, grafting of mcl-PHA with CMCH (CMCH-*g*-mcl-PHA) was successfully achieved in this study. Mcl-PHA used for the synthesis of graft was extracted from *C. testosteroni* grown on coconut oil.[8] CMCH was synthesized from chitosan as per the procedure described in Materials and methods. The graft was synthesized using CAN as an initiator. Very few studies on grafting of PHA with chitosan have been carried out. Yalpani et al.[14] studied the grafting of PHB onto chitosan, and Arslan et al.[4] developed graft of poly(3-hydroxyoctanoate) onto chitosan via condensation reactions between carboxylic acids and amine groups.

Fourier transform infrared spectroscopy

FTIR spectrum of mcl-PHA (Fig. 1) obtained from *C. testosteroni* during growth on coconut oil showed the presence of following groups: IR (KBr): 3465 cm^{-1} (vw, —OH), 2955.75 cm^{-1}, 2926.51 cm^{-1}, 2856.79 cm^{-1} (s, —CH3 stretching), 1743.01 cm^{-1} (versus, C=O of ester group), 1466.26 cm^{-1} (ms, δ_{as} CH_3/ δCH_2), 1378.94 cm^{-1} (ms, δ_sCH3), and 1169.35 cm^{-1} (s, vC—O).[8]

The IR spectrum of CMCH (Fig. 2) showed the strong peak at 1412.3 cm^{-1}, which could be assigned to the symmetrical stretching vibration of COO^-. The asymmetrical stretching vibration of COO^- (1900–1550 cm $^{-1}$) overlapped with the deforming vibration of NH_2 at 1599.3 cm^{-1} giving a very strong peak. The C—O absorption peak of secondary hydroxyl group is observed at 1074 cm^{-1}.

FTIR spectrum of CMCH-*g*-mcl-PHA (Fig. 3) showed the IR peaks at 2926.10 cm^{-1}, 2856.08 cm^{-1}, 1739.22 cm^{-1}, 1573.24 cm^{-1}, 1410.37 cm^{-1}, 1314.28 cm^{-1}, 1166.82 cm^{-1}, 1071.55 cm^{-1}, and 555.04 cm^{-1}. The absorption at 1573.24 cm^{-1} and 1410.37 cm^{-1} was attributed to deforming vibration of NH_2 and COO^-, respectively. The presence of absorption band at 1071.55 cm^{-1} is attributed to C—O absorption as observed in CMCH. Moreover, the absorption at 2926.10 cm^{-1} and 2856.08 cm^{-1} can be attributed to —CH_3 stretching. About 1739.22 cm^{-1} shows a very strong C=O stretching. Thus, IR

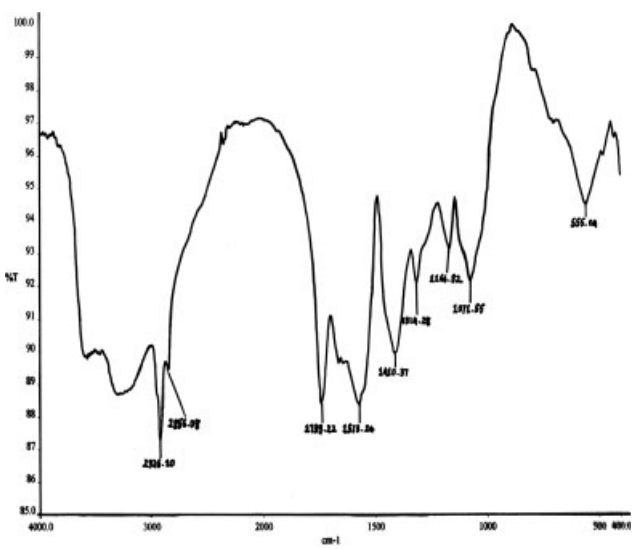

Figure 3 FTIR of CMCH-*g*-mcl-PHA.

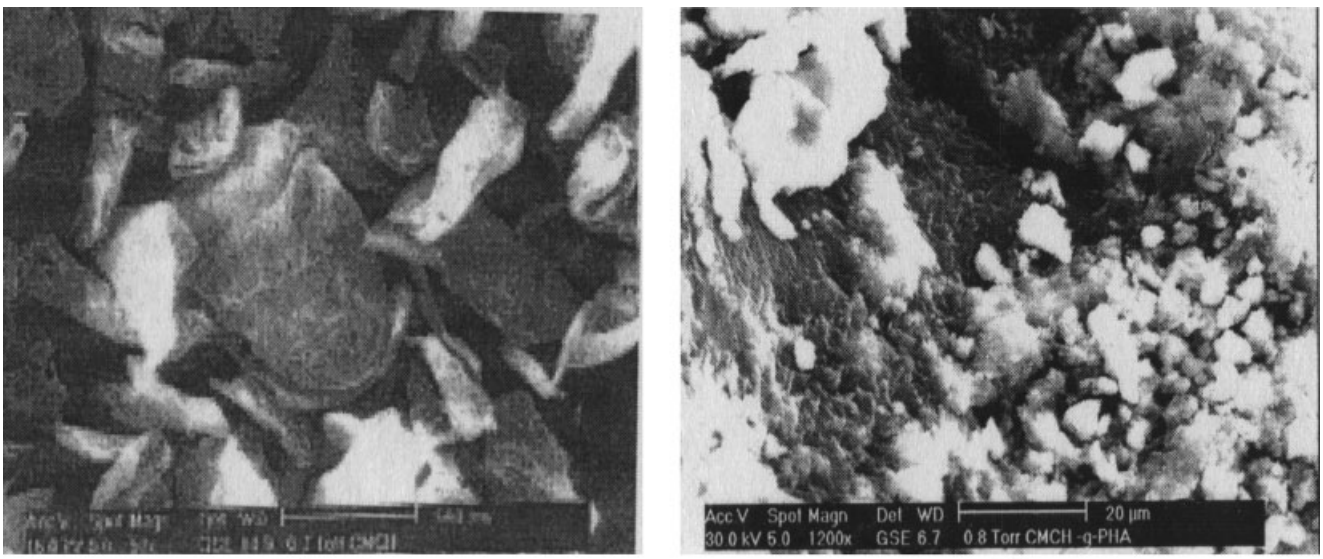

Figure 4 (a) Scanning electron micrograph of CMCH. (b) Scanning electron micrograph of CMCH-*g*-mcl-PHA.

analysis of the graft confirms the successful graft copolymerization between mcl-PHA and CMCH.

Scanning electron microscopy

The scanning electron micrographs of CMCH and its graft copolymer are shown in Figure 4(a,b). Graft copolymerization has affected the surface morphology and also physical and chemical properties of CMCH. It is clearly seen from SEM that the fibrous nature of CMCH is modified in the grafted product. The slightly flaky appearance of CMCH [Fig. 4(a)] is modified to the clustered irregular structure in CMCH-*g*-mcl-PHA [Fig. 4(b)]. Similar observation has been made by Joshi and Sinha,[10] wherein the fibrous nature of CMCH is modified due to grafting of methacrylic acid onto CMCH.

Thermogravimetric analysis

The thermogram of the grafted polymer exhibits mainly two decomposition temperatures; one at 100°C due to loss of associated moisture and the other at around 380°C due to the decomposition of the graft with around 50% loss in weight at 400°C. Comparing the thermograms of mcl-PHA [Fig. 5(a)] with CMCH-*g*-mcl-PHA [Fig. 5(c)], it is evident that grafting has decreased the thermal stability of the polymer, because mcl-PHA was showing $\sim$ 4.39% weight loss at 233.15°C, whereas the polymeric graft showed almost 23–24% loss in weight at the same temperature. Similar observations on decreased thermal stability have been reported by Arslan et al.,[4] whereas grafting PHO onto chitosan. In contrast, on comparing thermogram of CMCH [Fig. 5(b)] with that of the graft, it was found that grafting of mcl-PHA onto CMCH has resulted in increased thermal stability of CMCH. Liang et al.[15] observed an increase in the thermal stability of the graft when (dimethylamino) ethyl methacrylate was grafted onto chitosan using CAN as an initiator.

Differential scanning calorimetric analysis

Differential scanning calorimetric (DSC) thermogram of mcl-PHA [Fig. 6(a)] depicted the thermal stability of native mcl-PHA between 230 and 240°C, which confirmed the results of TGA analysis. The melting point of mcl-PHA was 50°C, which was similar to the reported T_m range between 39 and 61°C.[1] On comparing DSC thermogram of mcl-PHA with that of the graft [Fig. 6(b)], a shift in the endotherm was observed from 50°C to 74.90°C, suggesting the melting temperature of the graft to be around 74.9°C.

Solubility studies of CMCH-*g*-mcl-PHA

Attempts were made to solubilize the graft in different solvents namely water, acetic acid, chloroform, formic acid, dichloromethane, hexane, ethyl acetate, *n*-butylacetate, isoamylalcohol, *n*-butanol, formaldehyde, triethanolamine, diethylamine, petroleum ether, glycerin, glycerol, phenol, gluteraldehyde, isopropanol, benzene, methanol, toluene, 1 to 4 wt % acetic acid, acetic acid : chloroform (1 : 1), acetic acid : acetone (1 : 1), acetic acid : ethanol (1 : 1), and dimethylsulfoxide. But the polymer did not show solubility in any of the solvents. Swelling of the

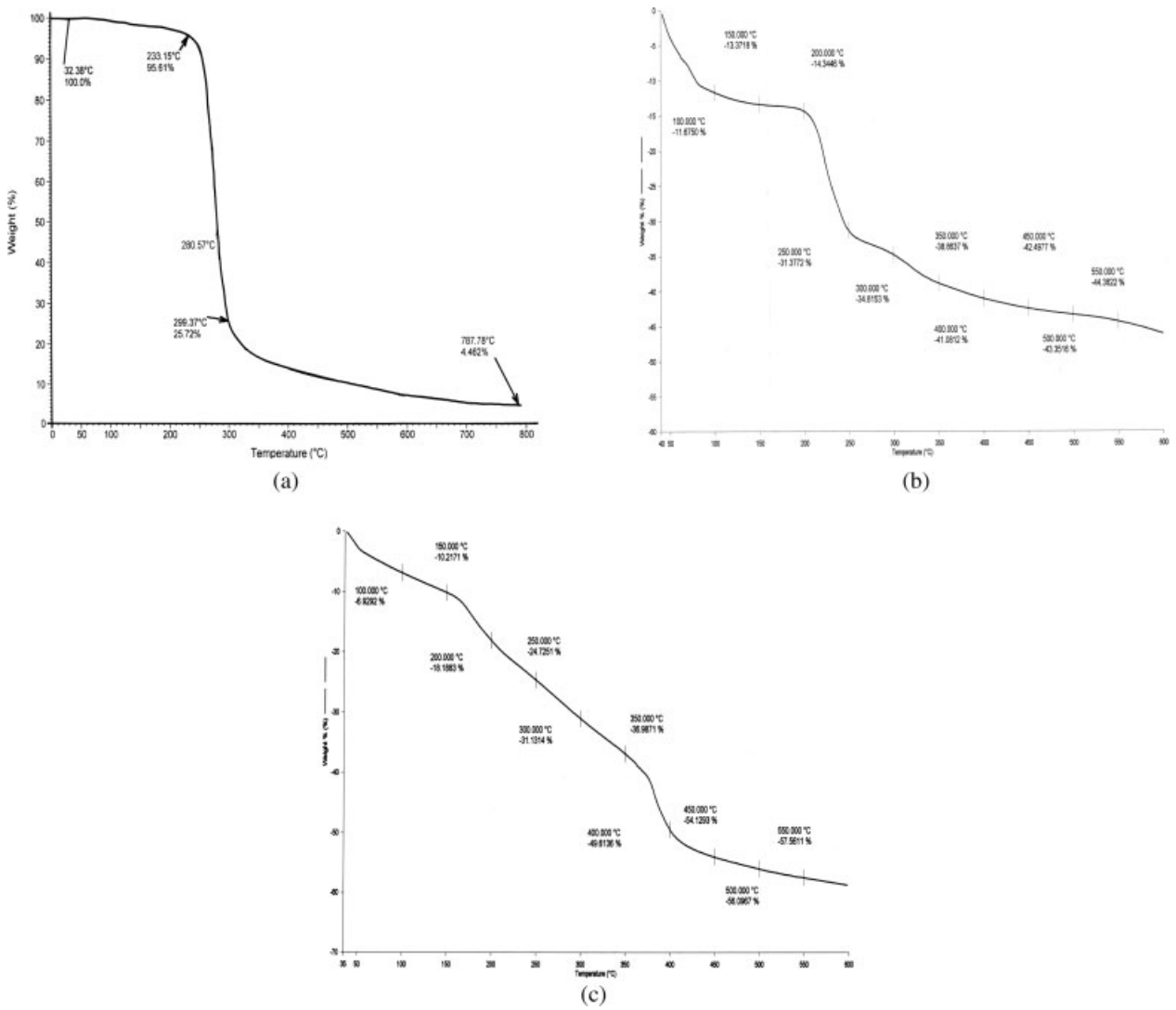

Figure 5 (a) Thermogravimetric analysis of purified mcl-PHA produced by *C. testosteroni* during cultivation on 1% coconut oil. (b) Thermogravimetric analysis of CMCH. (c) Thermogravimetric analysis of CMCH-*g*-mcl-PHA.

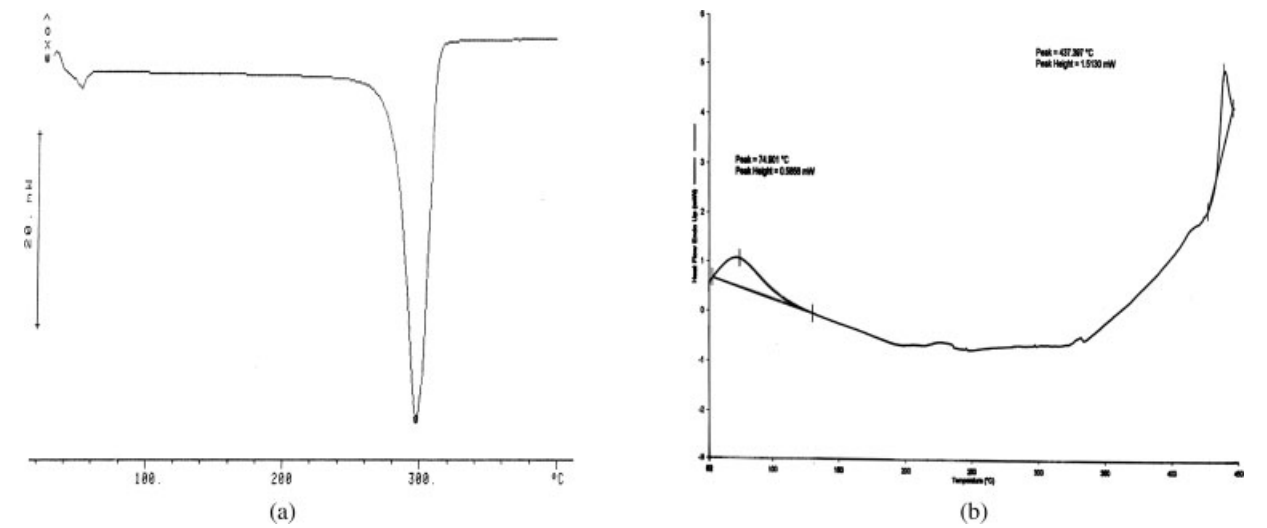

Figure 6 (a) Differential scanning calorimetric analysis of mcl-PHA produced by *C. testosteroni* during cultivation on 1% coconut oil. (b) Differential scanning calorimetric analysis of CMCH-*g*-mcl-PHA.

TABLE I
Percentage Weight Loss of the Polymer

Microbial culture	Polymer degradation in terms of % weight loss	
	Shaking	Static
	CMCH	
Aspergillus fumigatus 202	97.82	98.64
Burkholderia cepacia 202	97.26	78.52
	CMCH-*g*-mcl-PHA	
Aspergillus fumigatus 202	93.26	51.10
Burkholderia cepacia 202	76.06	70.30
	PHA	
Aspergillus fumigatus 202	10.00	04.00
Burkholderia cepacia 202	16.00	18.00

polymeric graft was observed in case of water, petroleum ether, and acetic acid. According to Arslan et al.,[4] the solubility of graft copolymers in 2 wt % acetic acid depends on the number of unreacted (free) NH_2 groups on CMCH backbone. When grafting percent increases with the decrease in the number of free NH_2 groups, solubility of the polymer decreases. This indicated the higher percent grafting in the graft.

Biodegradation studies of CMCH-*g*-mcl-PHA

The research on biodegradability of degradable plastics has become one of the focuses of advanced material researching. PHAs are well-known biodegradable plastics. Because PHAs are abundant in environment, a wide range of bacteria and fungi have developed the capability to use them. It has been shown that PHA degradation occurs in a large variety of complex ecosystems, including oxic and anoxic environment such as soil, seawater, and sludge.[16] CMCH is a biodegradable polymer derived from chitin through chitosan.[5] It possess —OH and —COOH, which are versatile groups responsible for the biodegradation. The graft synthesized from both these biodegradable material may also be biodegradable and eco-friendly. Moreover, CMCH-*g*-mcl-PHA was insoluble in various solvents and had high-thermal stability, which proved it to be an industrially viable polymer. Hence, its biodegradability was checked using a bacterial culture *B. cepacia* 202 and a fungal culture *A. fumigatus* 202.

For biodegradation of any polymer, availability of the polymer to the microorganism degrading it and oxygen concentration are two most important factors. Hence, we carried out the biodegradation studies in static as well as shaking conditions. The biodegradation of CMCH-*g*-mcl-PHA was studied in terms of increase in growth of the organism, extracellular protein concentration in the medium, and decrease in the % initial weight of the graft. Because the graft was provided as the sole source of carbon for the growth of the organisms, the increase in growth proved to be an indirect indication for the biodegradation of the graft.

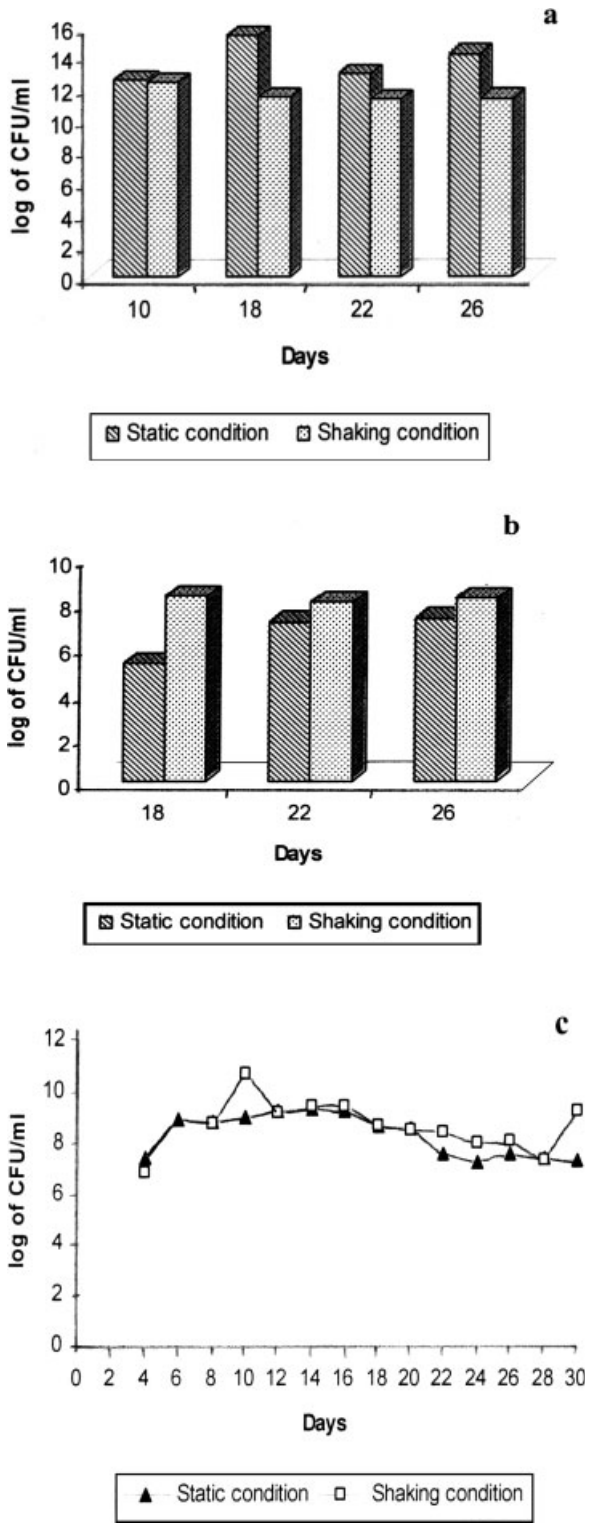

Figure 7 Growth of *Burkholderia cepacia* 202: (a) on mcl-PHA as sole carbon source, (b) on CMCH as sole carbon source, (c) on CMCH-*g*-mcl-PHA as sole carbon source.

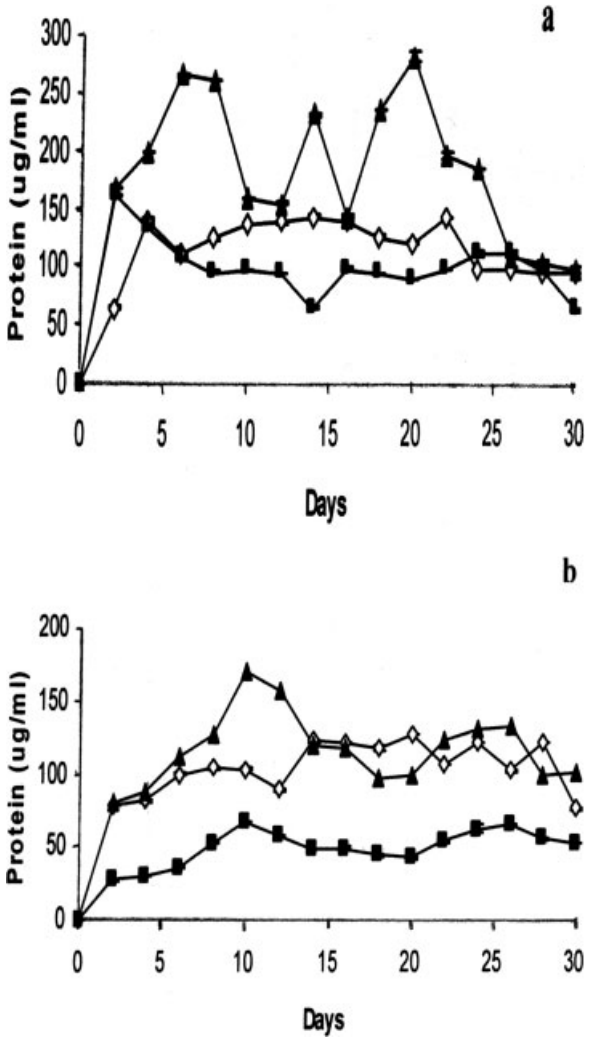

Figure 8 Extracellular protein concentration obtained due to degradation of CMCH-*g*-mcl-PHA by *Burkholderia cepacia* 202. —◇— CMCH-*g*-mcl-PHA, —■— PHA, and —▲— CMCH. (a) Under shaking conditions and (b) under static conditions.

As seen in Table I among the three polymers CMCH, mcl-PHA, and CMCH-*g*-mcl-PHA; weight loss of CMCH was maximum followed by that of graft, and least weight loss was observed in pure mcl-PHA samples. CMCH, derived from chitosan, is highly susceptible to degradation. This could be the reason for highest degradation of CMCH (98% weight loss) both by fungal and bacterial cultures. CMCH-*g*-mcl-PHA was also degraded by both fungus and bacteria. But the extent of degradation was lesser than pure CMCH. This might be attributed to the insertion of PHA onto CMCH, which would have resulted into blockage of few carboxyl groups where PHA might have been grafted. Least degradation, ranging from 4 to 18% weight loss was observed in case of PHA, this might be due to presence of long aliphatic chains in the structure of mcl-PHA with one free —COOH and one free —OH groups at the end of the chains. Similar results of degradation of mcl-PHA by *B. cepacia* 202 was observed earlier by us.[13] From the results of % weight loss, it can be concluded that the static condition is favorable for the degradation, because not much difference in terms of % weight loss was observed in case of static as well as shaking conditions during degradation (Table I).

The increase in growth of the *B. cepacia* 202 using mcl-PHA, CMCH, and CMCH-g-mcl-PHA was checked in terms of CFU/mL at an interval of 2 days, and the results [Fig. 7(a–c)] showed that the polymers supported the growth of *B. cepacia* 202 during its degradation.

Along with the weight loss, extracellular protein content was also determined. The protein secreted by the microorganism during its growth on polymer may comprise the several enzymes such as mcl-PHA depolymerase, chitosanase, hydrolase, and esterase. The increase in the protein concentration up to 239.44 μg/mL in case of *B. cepacia* 202 [Fig. 8(a,b)] and 236.98 μg/mL in case of *A. fumigatus* 202

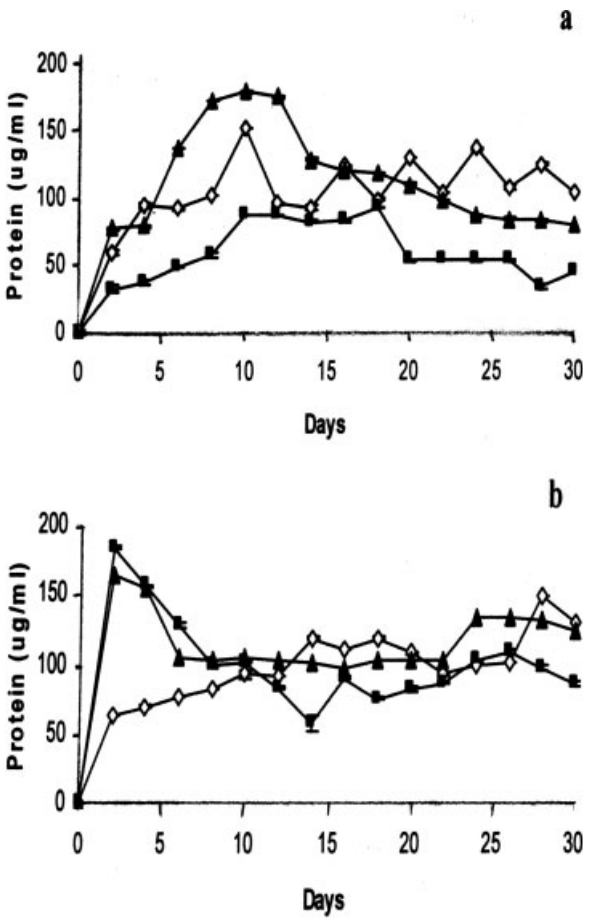

Figure 9 Extracellular protein concentration obtained due to degradation of CMCH-*g*-mcl-PHA by *Aspergillus fumigatus* 202. —◇— CMCH-*g*-mcl-PHA, —■— PHA, and —▲— CMCH. (a) Under shaking conditions and (b) under static conditions.

[Fig. 9(a,b)] was observed. This increase also proved the degradation of the polymer, because the polymer was the sole source of carbon for the growth of the organisms.

The extent of biodegradation depends on several factors such as temperature, pH, water potential, oxygen content, stereoregularity and crystallinity of the polymer, and its material processing. Because the studies were carried out in BHMs medium, the degradation obtained might be below maximum, which can be improved by optimizing culture conditions and media components, which could lead to more degradation within lesser incubation time.

CONCLUSIONS

Mcl-PHA obtained was soft, elastomeric, and slightly sticky material, and so it was difficult to handle. Grafting of this mcl-PHA with CMCH was carried out with CAN as an initiator. IR analysis verified the presence of mcl-PHA onto CMCH resulting in successful grafting. Changes in the morphology of the polymer, due to grafting reaction, were observed by SEM. Thermogram of the graft revealed its possible applications at higher temperatures. The graft served as a sole source of carbon for the growth of a bacterial isolates *B. cepacia* 202 and a fungal isolate *A. fumigatus* 202, which proved it to be biodegradable. Being synthesized from biocompatible polymers, CMCH-*g*-mcl-PHA has possible applications in the field of medicine such as tissue engineering and drug-delivery systems. The synthesized graft also has higher temperature resistance, and thus it possess advantage of both natural and synthetic polymer.

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